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Capillary electrophoresis of the mycotoxin zearalenone using cyclodextrin-enhanced fluorescence☆

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Abstract

Certain of the cyclodextrins are capable of significantly enhancing the native fluorescence of the estrogenic mycotoxin zearalenone (ZEN). Twenty-two cyclodextrins (CDs) were screened for their ability to enhance the fluorescence of ZEN in a capillary electrophoresis-laser induced fluorescence (CE-LIF) format. Of the CDs that were examined heptakis (2,6-di-*O*-methyl)-β-CD gave the greatest enhancement. The heptakis (2,6-di-*O*-methyl)-β-CD was applied to the development of a CE-LIF method for detection of ZEN in maize. The resulting method was capable of detecting ZEN with a limit of quantitation of 5 ng/g maize. Recoveries of ZEN from maize spiked over the range from 5 ng/g to 500 ng/g averaged 103.1 ± 8.5% ($n = 20$). The CE-LIF method will be useful for future studies of ZEN in maize.

Keywords: Capillary electrophoresis; Zearalenone; Mycotoxins; Cyclodextrins; Maize

1. Introduction

Zearalenone (ZEN, Fig. 1) is a mycotoxin produced by several fungi including *Fusarium graminearum* (sexual state *Gibberella zeae*). This mycotoxin is a frequent contaminant of maize, barley, and other grains throughout the world [1]. While the acute toxicity of ZEN is relatively low, ZEN and related compounds such as α- and β-zearalenol have potent estrogenic activities. A third analog of ZEN, α-zearalanol (also known as zeranol), has anabolic effects and is used in some countries as a growth promoter in cattle. The estrogenic activities of ZEN and related compounds have been reviewed [2].

Because of the potential health effects of ZEN and its congeners in domestic animals (particularly swine) human food and animal feed are frequently tested for the presence of this toxin. While the US Food and Drug Administration has not established a guideline for an acceptable level of ZEN, several other nations have established maximum tolerances ranging from 50 μg/kg to 1000 μg/kg in foods [3]. The World Health Organization in 2000 established a provisional maximum tolerable daily intake (PMTDI) for ZEN of 0.5 μg/kg body weight [1]. Therefore, testing methods for ZEN should be capable of detecting this toxin at, or below, 50 μg/kg in foods.

Detection methods for ZEN in foods are varied and include all of the commonly used chromatographic techniques, hyphenated techniques, and immunoassays. Instrumental methods for detection of ZEN were recently reviewed [4–6]. The most widely used instrumental methods include HPLC with either ultraviolet (UV) or fluorescence detection [7–9], HPLC with mass spectrometric detection [10–12], and gas chromatography with mass spectrometric detection [13]. Widely used for screening are the immunoassays, and immunosensors have also been recently reported [14–17].

Cyclodextrins (CDs) have been shown previously in a number of systems to enhance fluorescence of hydrophobic fluorophores. An example is with certain of the aflatoxin mycotoxins, the fluorescence of which have been enhanced significantly through the addition of CDs [18]. This property has proven useful in improving the detection limits for aflatoxin in capillary electrophoresis (CE) systems with laser induced fluorescence detection (CE-LIF) [19]. β-Cyclodextrin has also been used as a mobile phase additive in an HPLC application for the

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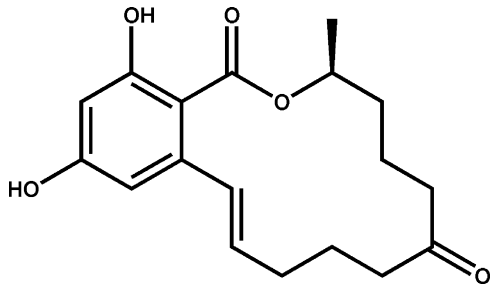

Fig. 1. Zearalenone {6-[(10*S*)-10-hydroxy-6-oxo-*trans*-1-undecenyl]-β-resorcylic acid lactone}.

separation of ZEN from another mycotoxin, ochratoxin A (OA) [7]. The latter report proposed the formation of an inclusion complex of ZEN with β-CD thereby effecting the chromatographic separation. However, the effect upon fluorescence intensity of ZEN caused by formation of the inclusion complex was not reported.

Capillary electrophoresis has also been used for ZEN detection [20–22]. The earliest report [20] investigated the use of α-, β-, an γ-CDs as modifiers of the mycotoxin retention, and described a strong interaction between γ-CD and ZEN. However, in that report UV detection (254 nm) rather than fluorescence was used, and the potential effect of the inclusion complex on ZEN fluorescence was not described. Böhs et al. [21] examined the effects of several CDs on the separation of mycotoxins, including ZEN. CDs examined included: β-CD, 2,3,6-methyl-β-CD, hydroxypropylated-β-CD (HP-β-CD), γ-CD, and HP-γ-CD. The apparent mobility of ZEN was reduced for the 2,3,6-methyl-β-CD and increased for the other CDs, relative to the mobility in the absence of a CD. The authors further suggested that, unlike OA, ZEN may be too large to form inclusion complexes with HP-β-CD. Although the authors examined several CDs, the method of detection was by photodiode array, and therefore potential effects of the CDs upon ZEN fluorescence were not reported.

From the literature it is clear that the cyclodextrins have the potential to influence the fluorescence of ZEN, although a systematic description of this phenomenon and the application to a CE method for ZEN have not been reported. The objective of this research was to examine a wide variety of cyclodextrins for the ability to enhance the fluorescence of ZEN using CE-LIF and, further, to develop a CE-LIF method with sufficient sensitivity to detect ZEN in maize at relevant levels.

2. Experimental

2.1. Chemicals and materials

Except where noted otherwise, deionized water (Nanopure II, Sybron/Barnstead) was used in the preparation of all reagents. All solvents were HPLC grade. ZEN was purchased from International Minerals & Chemicals (Terre Haute, IN, USA). Cyclodextrins were either purchased from Sigma (St. Louis, MO, USA) or synthesized at the USDA-NCAUR (Peoria, IL, USA). Purchased CDs included: α-CD, methyl-α-CD, heptakis(2,3,6-tri-*O*-methyl)-α-CD, (2-hydroxy)propyl-α-CD, carboxymethylated α-CD, carboxyethylated α-CD, α-CD phosphate (sodium salt), β-CD, heptakis(2,6-di-*O*-methyl)-β-CD, heptakis(2,3,6-tri-*O*-methyl)-β-CD, (2-hydroxy)propyl-β-CD, carboxymethylated β-CD, carboxyethylated β-CD, β-CD phosphate (sodium salt), 6-monodeoxy-6-monoamino β-CD, γ-CD, succinyl-γ-CD, carboxymethylated γ-CD, and carboxyethylated γ-CD. Mono-6-*N*-allylammonium-6-deoxy-β-CD chloride was prepared as previously described [23]. All other chemicals and solvents were reagent grade or better and purchased from major suppliers.

2.2. Apparatus

The instrument consisted of a Beckman Coulter P/ACE MDQ capillary electrophoresis system equipped with a dual laser induced fluorescence (LIF) detector (Beckman Coulter, Fullerton, CA, USA). A fused silica capillary, 100 μm i.d., 50 cm length to detector (60.2 cm overall length) maintained at 30 °C, was used for all experiments. All experiments were conducted in the "normal" electrophoretic mode, with the cathode at the outlet of the capillary. Excitation light was provided by a model 100 He/Cd laser at 325 nm (Omnichrome, Chino, CA, USA). Emission light was filtered through a 470 nm bandpass filter (Andover Corporation, Salem, NH, USA) and data were collected using Beckman Coulter 32 Karat software at a rate of 16 Hz.

2.3. Screening of cyclodextrins for fluorescence enhancement

Twenty-two cyclodextrins were assayed by CE-LIF to determine their suitability for enhancement of ZEN fluorescence. The electrophoresis buffer consisted of either 20 mM sodium borate adjusted to pH 8.5 (BB), or BB with the indicated cyclodextrin added at a level of 2 mg/ml. The decision to use a mass (rather than a molar) basis for preparing the cyclodextrin solutions was based upon the lack of available molecular or formula weights for most of the cyclodextrins tested. Before each injection the capillary was rinsed with electrophoresis buffer for 1 min at 20 psi. ZEN standard at a concentration of 10 μg/ml in deionized water was injected by applying 0.5 psi for 7 s. The separation was initiated by the application of 20 kV for 8 min. The capillary was rinsed for 1 min at 20 psi with 0.25 M sodium hydroxide and for 1 min at 20 psi with deionized water. Including the capillary washes before and after the sample separation and the separation itself, each assay took 12 min to perform.

2.4. Extraction of maize samples and isolation of ZEN

Maize was ground to a fine consistency using a coffee grinder. Maize tested and found to contain less than 2 ng ZEN/g, control maize, was spiked with ZEN to give levels ranging from 5 ng/g to 500 ng/g. ZEN stock solution (0.1 ml in acetonitrile/water) was added to 25 g of control maize and held overnight at ambient temperature to dry before extraction. In addition, a small number of samples naturally contaminated with ZEN were also tested. ZEN was extracted from the maize samples by shak-

ing on a wrist action shaker (Burrell, Pittsburgh, PA, USA) for 90 min with 125 ml acetonitrile/water (84/16, v/v). The extract was passed through a 2 V filter (Whatman, Maidstone, UK) and 10 ml was diluted with 25 ml of phosphate buffered saline (PBS: 10 mM sodium phosphate, 145 mM sodium chloride, pH 7.2). The diluted extract was passed through a 0.2 μm nylon membrane (Alltech, Deerfield, IL, USA) and 14 ml, equivalent to 0.8 g maize, was applied to an immunoaffinity column (Zearalenetest, Vicam, Watertown, MA, USA) at a flow rate of 1 drop/s. The column was washed with 5 ml deionized water, and the ZEN was eluted with 3 ml acetonitrile into a silane-treated vial. The purified extract was dried under a gentle stream of nitrogen at 50 °C. The dried extract was reconstituted with 0.2 ml acetonitrile and 0.6 ml water, then passed through a 17 mm syringe filter (0.2 μm polyvinylidene fluoride membrane, Alltech) before injection onto the CE.

2.5. CE-LIF assay for ZEN in maize

For testing of maize samples an optimized form of the CE-LIF screening assay was used. The electrophoresis buffer was 20 mM sodium borate, pH 8.5, with sodium deoxycholate and heptakis(2,6-di-*O*-methyl)-β-CD added to obtain final concentrations of 1 mM and 2 mM respectively. The voltage applied was 10 kV, all other conditions were as described above for the screening assay. Concentrations of ZEN in spiked or naturally contaminated maize were calculated from the sample responses relative to the linear regression fit of ZEN standards over the range of 5–1000 ng/ml.

3. Results and discussion

3.1. Screening of cyclodextrins for fluorescence enhancement

Previous literature has indicated the potential enhancement of the fluorescence of mycotoxins such as the aflatoxins by cyclodextrins [18,19], and cyclodextrins have been used to improve the separation of zearalenone by CE [20,21] and HPLC [7]. Although the effects on reverse-phase chromatography were reported [7], the enhancement of ZEN fluorescence with cyclodextrins was not. This is not entirely surprising, because the fluorescence of ZEN is environmentally sensitive, and the HPLC method used a mobile phase of sufficient solvent strength (45% methanol) that the cyclodextrins may not have significantly enhanced the fluorescence under those conditions. The CE method we report here is aqueous based, which allowed us to observe an effect that may have been masked by solvent effects in the previous HPLC report. In addition, the few reported CE methods for ZEN have used UV or photodiode array (PDA) detection, which would likewise have kept the investigators from observing the effect of the CDs upon the fluorescence intensity [20–22]. As a result, the effects of CDs upon the fluorescence intensity of ZEN have not been previously reported.

We have endeavored to conduct a systematic examination of cyclodextrins to determine which modifications would provide the greatest enhancement of ZEN fluorescence. In order to maximize fluorescence intensity of ZEN 22 CDs were screened for their ability to enhance ZEN fluorescence in a CE-LIF application. Twenty-one of these CDs are commercially available and one was synthesized at our facility. The CE-LIF screening assay used to evaluate the CDs was conducted with a fairly high concentration of ZEN (10 μg/ml). This was necessary in order for the native fluorescence of ZEN, which is relatively weak in aqueous solutions, to be observed. The CDs were evaluated for their effects upon the migration of the ZEN peak as well as upon the peak area and height (Table 1).

In Table 1 the effects upon ZEN fluorescence are presented as the ability to enhance fluorescence, calculated by taking the ratio of the fluorescence peak height (or area) in the presence of CD to the peak height (or area) in the absence of CD. Most of the α-CDs showed minimal effects upon ZEN fluorescence, whether measured by peak height or peak area. However, several of the β- and γ-CDs showed substantial effect. The parameter used for evaluation (peak height or area) was important. When peak height was used for evaluation, the greatest enhancement was seen with the heptakis (2,6-di-*O*-methyl) derivative of β-CD. When peak area was used for evaluation, the greatest enhancement was seen with the carboxyethylated β-CD followed by the di-methyl derivative. The difference between the two methods of evaluation is likely related to peak shape. The peak shapes were dependent upon the physical properties of the CDs and the electrophoretic conditions used. Under the conditions used here the heptakis (2,6-di-*O*-methyl)-β-CD reduced the ZEN retention while the carboxyethylated β-CD increased ZEN retention relative to the absence of CD. The overall effect was that the ZEN peaks obtained with carboxyethylated β-CD were broader and shorter than ZEN peaks obtained with the di-methyl (Fig. 2).

It is apparent that, of the α-, β- and γ-CDs, which are composed of 6, 7, and 8 amylose residues respectively, the β-CDs in general provided the greatest enhancement of ZEN fluorescence, suggesting that the cavity formed by 7 amylose residues is the optimum for binding ZEN. Within the β-CD series, the unmodified β-CD was one of the most effective. Modifications that rendered the CD less hydrophilic, tended to enhance fluorescence. Modifications that rendered the CD more hydrophilic tended to reduce fluorescence. Clearly, even minor changes to the structure, such as the difference between the (2,6-di-*O*-methyl) and the (2,3,6-tri-*O*-methyl) were capable of producing dramatic differences in the ability of the CDs to interact with the ZEN and thereby enhance fluorescence. A more mechanistic study of the interactions between ZEN and the CDs will be the subject of a future manuscript.

3.2. CE-LIF assay for ZEN in maize

To develop a CE-LIF assay sensitive enough to detect ZEN at relevant levels in maize (at or below 50 μg/kg), we chose to use the CD with the greatest enhancement of ZEN fluorescence that was readily available commercially. Because heptakis (2,6-di-*O*-methyl)-β-CD is readily available and enhanced the peak height by approximately 20-fold (Table 1), this derivative was chosen for further development.

Table 1
Effects of 22 cyclodextrins on zearelenone (ZEN) fluorescence

Cyclodextrins	Modification of cyclodextrin[a]	Migration time (min)	Relative enhancement of ZEN response[b]	
			By peak area	By peak height
No cyclodextrin		5.5	1.0	1.0
α-Cyclodextrins	Unmodified	5.5	1.0	1.0
	Methyl	5.4	1.2	1.3
	Heptakis(2,3,6-tri-*O*-methyl)	5.2	1.4	1.5
	(2-Hydroxy)propyl	5.0	1.2	1.2
	Carboxymethylated	5.8	1.1	1.0
	Carboxyethylated	5.5	1.3	1.3
	Phosphate	5.5	1.1	1.0
β-Cyclodextrins	Unmodified	4.8	7.3	10.7
	Heptakis (2-*O*-methyl)	5.1	6.3	7.5
	Heptakis (2,6-di-*O*-methyl)	4.4	10.4	19.7
	Heptakis (2,3,6-tri-*O*-methyl)	5.2	2.5	2.6
	(2-Hydroxy)propyl	4.6	7.0	9.3
	Carboxymethylated	6.0	6.0	4.4
	Carboxyethylated	5.8	11.4	8.5
	Phosphate	6.5	5.5	3.1
	Sulfate	5.6	1.3	1.3
	6-Monodeoxy-6-monoamino	4.6	7.1	11.1
	6-*N*-Allylammonium-6-deoxy	4.8	6.3	7.0
γ-Cyclodextrins	Unmodified	5.0	3.9	4.7
	Succinyl	5.0	2.8	2.9
	Carboxymethylated	6.7	3.9	1.8
	Carboxyethylated	6.2	4.2	3.0

[a] Chemical modification of the indicated cyclodextrin backbone.
[b] Calculated as the response with 2 mg/ml added cyclodextrin divided by the response without added cyclodextrin. Data are the averages of triplicate determinations, with the exception of the control (no cylcodextrin, $n = 31$), unmodified β-CD ($n = 6$), and heptakis (2,6-di-*O*-methyl)-β-CD ($n = 15$).

There is a substantial literature on effective cleanup techniques for isolating ZEN from foods. Acetonitrile mixtures with water, generally in the proportion of 80–90% acetonitrile, are most commonly used. Cleanup techniques can vary depending upon the type of instrumentation used in the analysis, but commonly used methods include charcoal-alumina cleanup columns and immunoaffinity columns (IAC). The charcoal-alumina columns retain sample matrix constituents and allow the ZEN to pass through, while the IAC retain the ZEN and therefore provide an additional opportunity to concentrate the toxin from the extract. In these experiments we spiked maize with ZEN, extracted with acetonitrile/water (84/16, v/v) and isolated the ZEN by IAC.

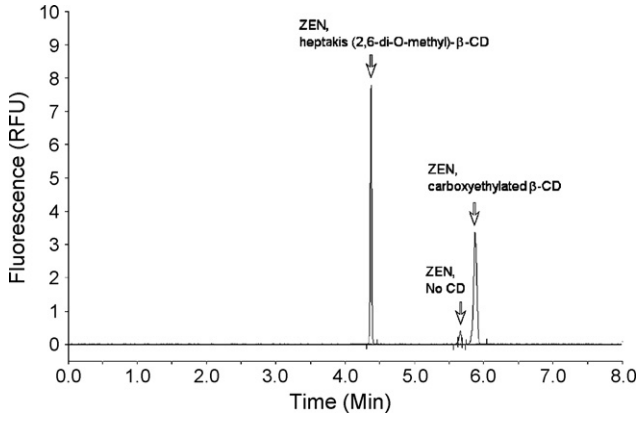

Fig. 2. Effects of two cyclodextrins upon zearalenone fluorescence and migration. Figure shows three overlaid electropherograms for ZEN in the presence of either: no added cyclodextrin (5.66 min) or the added cyclodextrins heptakis (2,6-di-*O*-methyl)-β-CD (4.38 min), or carboxyethylated β-CD (5.88 min).

The IAC cleanup gives a very clean extract, with few fluorescent interferences when tested by HPLC [8,16]. Because of the relatively high sensitivity required for the CE application, we decided to include an "on-capillary" pre-concentration step. The purified extract, in 25% (v/v) acetonitrile/water was injected under pressure. The electrophoresis buffer (EB) contained 20 mM sodium borate, 1 mM sodium deoxycholate, and 2 mM of the heptakis (2,6-di-*O*-methyl)-β-CD. The lower ionic strength of the sample buffer relative to the EB was designed to allow for sample stacking to increase sensitivity. In addition, the combination of the deoxycholate and CD in the EB were designed to allow further focusing of the ZEN ("sweeping"). The combination was adapted from a strategy known as "dynamic pH junction-sweeping" as described by Britz-McKibbin et al. [24] for the on-line pre-concentration of steroids.

The result was a very sharp ZEN peak (Fig. 3). The shape of the peak was useful in the separation of ZEN from potential interferences and in enhancing the assay sensitivity. The sharpness of the ZEN peak necessitated that the data collection rate for the detector be set at the highest setting (16 Hz), in order to ensure that the entire peak was captured. The limit of detection of the method, defined as a signal to noise ratio of 3 was

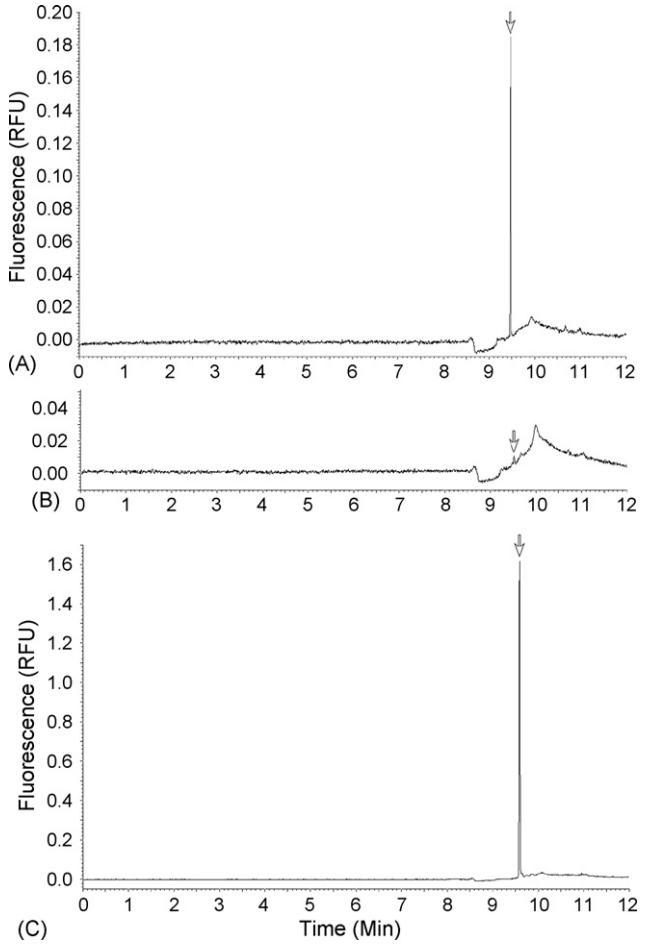

Fig. 3. Electropherograms of: (A) maize spiked with 50 ng zearalenone/g maize, (B) unspiked control maize, (C) maize naturally contaminated with ZEN at a level of 417 ng/g.

1 ng ZEN/ml. The maize used for the spiking studies contained a peak with a migration time similar to ZEN near the limit of detection, indicating the "control" maize may have had 1 ng ZEN/g maize (Fig. 3b).

Uncorrected recoveries from 5 ng/g up to 500 ng/g averaged 103.1 ± 8.5% (Table 2). The recoveries decreased slightly between 5 ng/g (109%) and 500 ng/g (97%), which may be due in part to the capacity of the IAC columns. Alternatively, the presence of up to 1 ng ZEN/g maize in the control maize may have artificially enhanced the recoveries for the lower spiking levels (5 ng/g and 10 ng/g, Table 2). If the control maize was assumed to contain 1 ng/g, then the recoveries at 5 ng/g and 10 ng/g would have been 90.8% and 101.4% (versus 109.4% and 111.6%), respectively. If the peak in the control maize was assumed to be ZEN, the overall recovery over the range of 5–500 ng/g would have been 96.8% after correction. The limit of quantitation (LOQ) of the method, defined as the lowest level that could be reliably quantitated, was 5 ng/g in maize. Extracts from samples containing greater than 500 ng ZEN/g maize may need to be diluted before application to the IAC.

Table 2
Recovery of zearalenone (ZEN) from spiked maize

Spiking level (ng/g)[a]	Average recovery (%)	Standard deviation (%)
5	109.4	11.2
10	111.6	6.5
50	100.7	4.0
200	96.8	1.9
500	96.9	3.9
Overall average	103.1	8.5

[a] Quadruplicate maize samples spiked at each level (overall $n = 20$).

4. Conclusions

Of the 22 CDs that were examined here for enhancement of ZEN fluorescence, the CD giving the greatest degree of enhancement, as measured by the effect upon height of the ZEN peak, was heptakis (2,6-di-*O*-methyl)-β-CD. Unmodified β-CD, 6-monodeoxy-6-monoamino-β-CD, and carboxyethylated-β-CD also substantially enhanced ZEN fluorescence. Of these the heptakis (2,6-di-*O*-methyl)-β-CD was chosen for use in a CE-LIF method for detection of ZEN in maize because it is available commercially in relatively large amounts. The resulting CE-LIF method was capable of detecting ZEN in maize over the range from 5 ng/g to 500 ng/g, and will be useful for future studies of this mycotoxin in maize.

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